

Supplementary Information

S1. General Remarks and Experimental for Bioassays

Biosafety approval was obtained from the Macquarie University Biosafety Committee (approval number 5201000870).

The strains of bacteria used were *Staphylococcus aureus* ATCC 9144 (obtained from the CDS Reference Laboratory, Department of Microbiology, The Prince of Wales Hospital, NSW, Australia), *Escherichia coli* JM109 and *Pseudomonas aeruginosa* ATCC 27853 (both obtained from Michael Gillings, Department of Biological Sciences, Macquarie University). Glycerol stock cultures (20% v/v glycerol) of these strains were kept at $-80\text{ }^{\circ}\text{C}$. Initial cultures were prepared by streaking a small quantity of the frozen stock onto an LB agar plate and incubation at $37\text{ }^{\circ}\text{C}$ for 16 h. The plates were stored at $4\text{ }^{\circ}\text{C}$ and used to inoculate subsequent cultures for up to one month. Overnight cultures were generally prepared by inoculating the according medium with a single bacterial colony from the agar plates, and subsequent incubation at $37\text{ }^{\circ}\text{C}$ for 16 h with shaking (100–150 rpm). The optical density at 600nm (OD600) was adjusted to 0.08–0.1 to produce an inoculum density of 1.0×10^8 cfu/mL (OD).

S1.1. Screening of Crude Partitions for Antimicrobial Activity

The weights of the investigated samples and resulting partitions are summarised in Table S1.

Table S1. Weights of investigated samples and of different extracts.

Sample ID	Initial Wet Weight (g)	Partition (Dried)			
		PE	EtOAc	BuOH	Water
JB07-S1	654	0.9076	6.5420	0.2241	5.7681
Mollusc	13	0.0401	0.0120	0.0956	0.1375

The preliminary antibacterial screening of the sponge extracts was performed against three human pathogenic microorganisms, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. These are some of the most frequently isolated pathogens from a variety of clinical conditions and are therefore ideal candidates to be used for screening antimicrobial agents [1].

S1.1.1. Disc Diffusion Test

The sponge extracts were screened in a disc diffusion assay against the three pathogens as part of a larger study looking at the geographic distribution of antimicrobial and herbicidal activity of sponge extracts. The assay is based on the Kirby-Bauer assay [2]. Here, a bacterial lawn is cultivated on an agar plate and a paper disc containing a known concentration of the test sample is placed on the surface. The assay relies on the ability of the test material to diffuse into the agar, and to prevent the growth of the organism. The zone of inhibition (ZOI) of growth of the organism is measured by the diameter of no growth (including the diameter of the disc, Ø 6 mm), indicating the efficacy of the test substance.

For the disc diffusion assay, the saturated overnight cultures were prepared in Luria Broth (LB) (15 mL). Sterile paper discs (Ø 6 mm) were impregnated with the extracts ($2 \times 25\text{ }\mu\text{L}$; 10 mg/mL 1:1 acetone:water), antibiotic (10 μL ; 1 mg/mL) or solvent control ($2 \times 25\text{ }\mu\text{L}$; 1:1 acetone:water) and dried hereafter. LB agarose medium (25 mL) was inoculated with the above inoculum (1 mL) at

45–50 °C, vortexed and poured (5 mL) onto fresh LB agar plates to give a thin layer (2 mm). The impregnated paper discs were pressed into the solidifying LB agarose layer. The plates were incubated at 37 °C for 16 h and the diameter of the ZOI was measured with a ruler. Measurements were rounded off to the next mm.

The ethyl acetate fraction of sponge JB07-S1 demonstrated the strongest level of antibacterial activity against *S. aureus* (ZOI: Ø 35 mm) and “moderate” against *E. coli* (Ø 11 mm) and no activity against *P. aeruginosa*.

S1.2. *Pseudoceratina purpurea*

Sample JB07-S1 was identified as *Pseudoceratina purpurea* (Carter 1880; family Pseudoceratinidae Carter; order Verongida; class Demospongiae) according to Bergquist *et al.* [3] and Hooper [4]. There are several synonymised taxa (*Aplysina purpurea*, *Dendrilla verongiformis*, *Druinella ramosa*, *Druinella tyroeis*, *Hexadella pleochromatum*, *Korotnewia desiderata*, *Psammaplysilla purpurea*, *Thorectopsamma xana*) [5].

The poriferan genus *Pseudoceratina* is characterised by a sparse fibrous skeleton organised on the dendritic plan, in which bark elements are absent. Its texture is firm, often hard and incompressible. Widely spaced tubercles or low raised ridges interrupt the otherwise smooth sponge surface. Its matrix is extremely dense and contains heavy collagen. As typical for all species of the Verongida order, *Pseudoceratina* changes colour upon death (yellow to deep purple).

S1.2.1. Bioassay-Guided Fractionation

The ethyl acetate partition was subjected to gel permeation filtration (in Sephadex). Correlating subfractions were combined and tested using the disc diffusion assay against the three bacterial strains. Sephadex fractions S-9-12, S-13-14 and S-15-22 all displayed some antibactericidal activity against *S. aureus* (Table S2) and were combined.

Table S2. The Sephadex fractions of JB07-S1 (*P. purpurea*) were tested for antibacterial activity in the disc diffusion assay and fractions showing activity are summarised below. Results are displayed in diameter (in mm) of ZOI.

<i>P. purpurea</i> EtOAc Partition Sephadex Fractions	Antibacterial Activity ^a in Disc Diffusion Test		
	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
(JB07-S1-EA-)S-9-12	7	14	-
(JB07-S1-EA-)S-13-14	8	16	-
(JB07-S1-EA-)S-15-22	8	10	-
Ampicillin	25	17	-
Chloramphenicol	-	-	20

^a The concentration of all samples was 30 µg/disc, except for ampicillin (10 µg/disc). The diameter of a disc is 6 mm.

S1.3. MTT Microdilution Assay

The purified bromotyrosine alkaloids from the ethyl acetate fraction of *P. purpurea* were subjected to antimicrobial MTT-microdilution assays [2] against *E. coli* and *S. aureus*. This colourimetric assay is based on the reductive cleavage of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by live cells to yield blue formazan. A colour change is an indicator of the presence of viable cells and lack of antiproliferative activity of the test sample.

All bacterial strains were grown overnight in MHB (15 mL). The assay was performed in sterile, clear flat-bottom-96-well microtitre plates. The arrangement of samples and controls was outlined according to Appendino *et al.* [6]. The pure HPLC-purified bromotyrosines (1.0 mg/mL) or the antibiotics (1 µg) were dissolved in DMSO (200 µL) and the final volume was made up to 1 mL with distilled water. Using a 96 well microtitre plate, MHB was dispensed into wells 1–11 (125 µL each) for each row, the extract or antibiotic solution was added to well 1 (125 µL; in different rows for each extract) and mixed thoroughly, after which 125 µL was taken out and dispensed to the next well (*i.e.*, well 2). This process of two-fold serial dilution was carried out until well 10, and skipping well 11, the final volume was dispensed into well 12. Again, 125 µL each of the bacterial inoculum was dispensed into wells 1 to 11 leaving well 12 free of inoculum. Well 11 was free of the test compound or the antibiotic, thus this acted as a positive growth control. Similarly, well 12 served as sterile control of the assay. 5% DMSO was also included as a negative control. The plate was incubated at 37 °C for 18 h. After incubation, 20 µL of a solution of MTT (5 mg/mL methanol) was added to each well and again incubated for 30 min. The MIC was determined as the lowest concentration of the test compound or antibiotic that showed no visible colour change from yellow to blue/purple.

MTT tests of **1–8** were performed as triplicates. The determined MICs are summarised in Table S3. Four of the tested brominated tyrosines (**2**, **6–8**) inhibited the growth of both *E. coli* and *S. aureus*. The other five compounds did not show any activity.

Table S3. MICs determined from MTT assay of HPLC purified bromotyrosines from *P. purpurea*.

Compound	MIC (µg/mL) ^a	
	<i>E. coli</i>	<i>S. aureus</i>
Aplysamine 8 (2)	125	31.3
Hexadellin (6)	>200 (n.r.)	>200 (n.r.)
Aplysamine-2 (7)	>200 (n.a. [7])	125 (n.a. [7])
16-debromoaplysamine-4 (8)	>200 (250 [8])	125 (200 [8])
Ampicillin	1.3 (0.25–1.0) ^b	1.1 (<0.25) ^b

^a MIC expressed as median of replicates ($n = 3$). Reference values in brackets; ^b Ampicillin control reference ranges [9]. n.r. = not reported. n.a. = not active.

S1.4. HR-LCMS Analysis

The combined bioactive Sephadex fractions of *P. purpurea* and the crude ethyl acetate partition of *T. corticalis* were analysed by HR-LCMS (Figure S1).

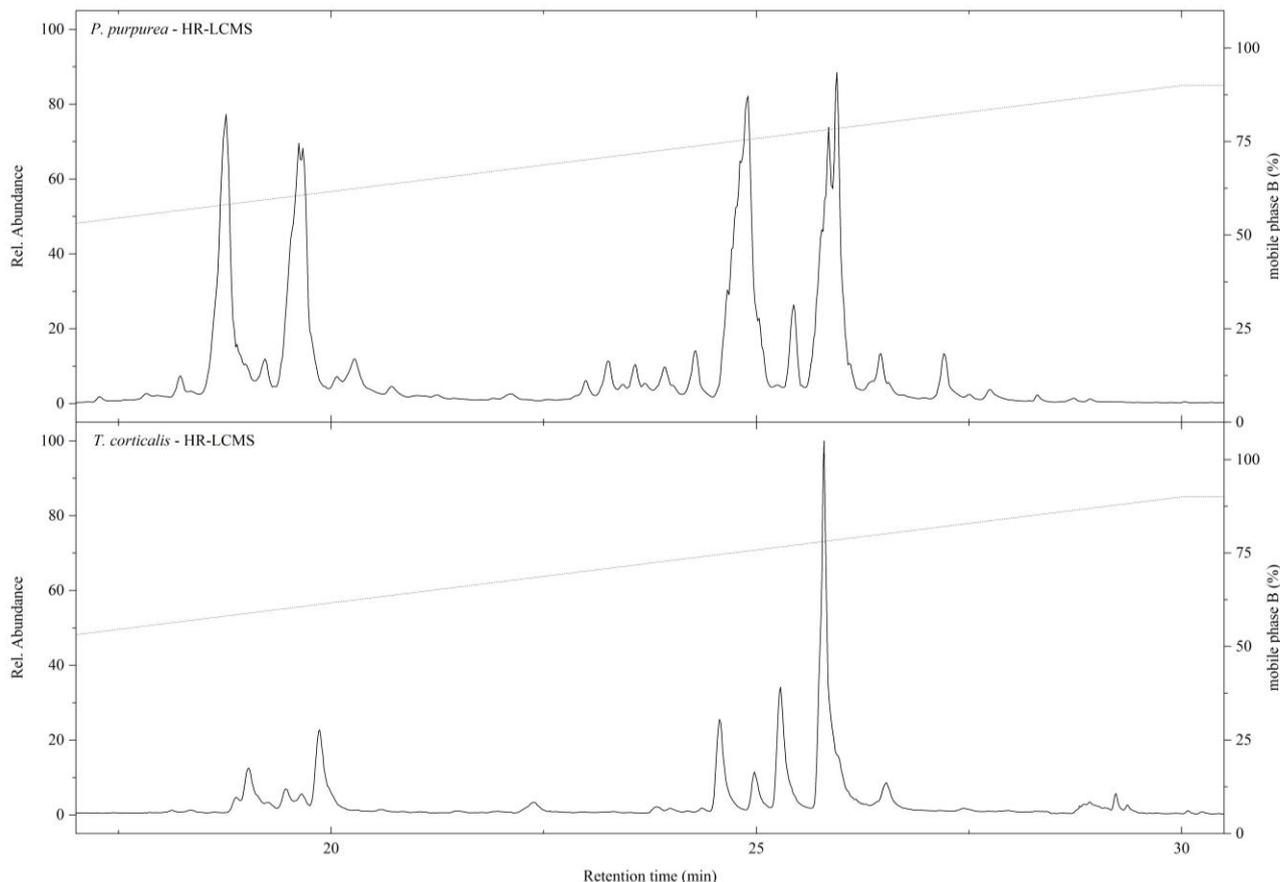


Figure S1. High resolution LC-MS chromatograms (TIC) of bioactive Sephadex fractions of ethyl acetate partitions of *P. purpurea* (JB07-S1-EA-S-9-22) and the crude EtOAc-partition of *T. corticalis*.

S1.5. Ceratinadin D (1)

The high resolution MS report for the new bromotyrosine derivative ceratinadin (**1**) is shown in Figure S2. The $^1\text{H-NMR}$ spectrum of (**1**) is shown in Figure S3.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 600.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

31 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-150 H: 0-250 N: 5-6 O: 6-8 Br: 2-2

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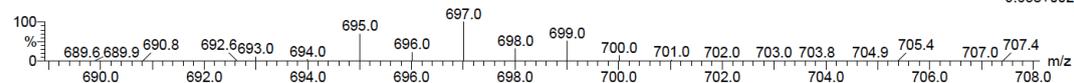
University of Illinois, SCS, Mass Spectrometry Lab

Qtof_36790 20 (1.436) AM (Cen,3, 80.00, Ar,15000.0,558.36,0.70,LS 3); Sm (SG, 2x3.00); Cm (18:20)

Q-tof UE521

1: TOF MS ES+

9.63e+002



Minimum: -1.5
Maximum: 5.0 10.0 600.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
695.0067	695.0101	-3.4	-4.9	15.5	25.6	C25 H25 N6 O8 Br2

Figure S2. Cont.

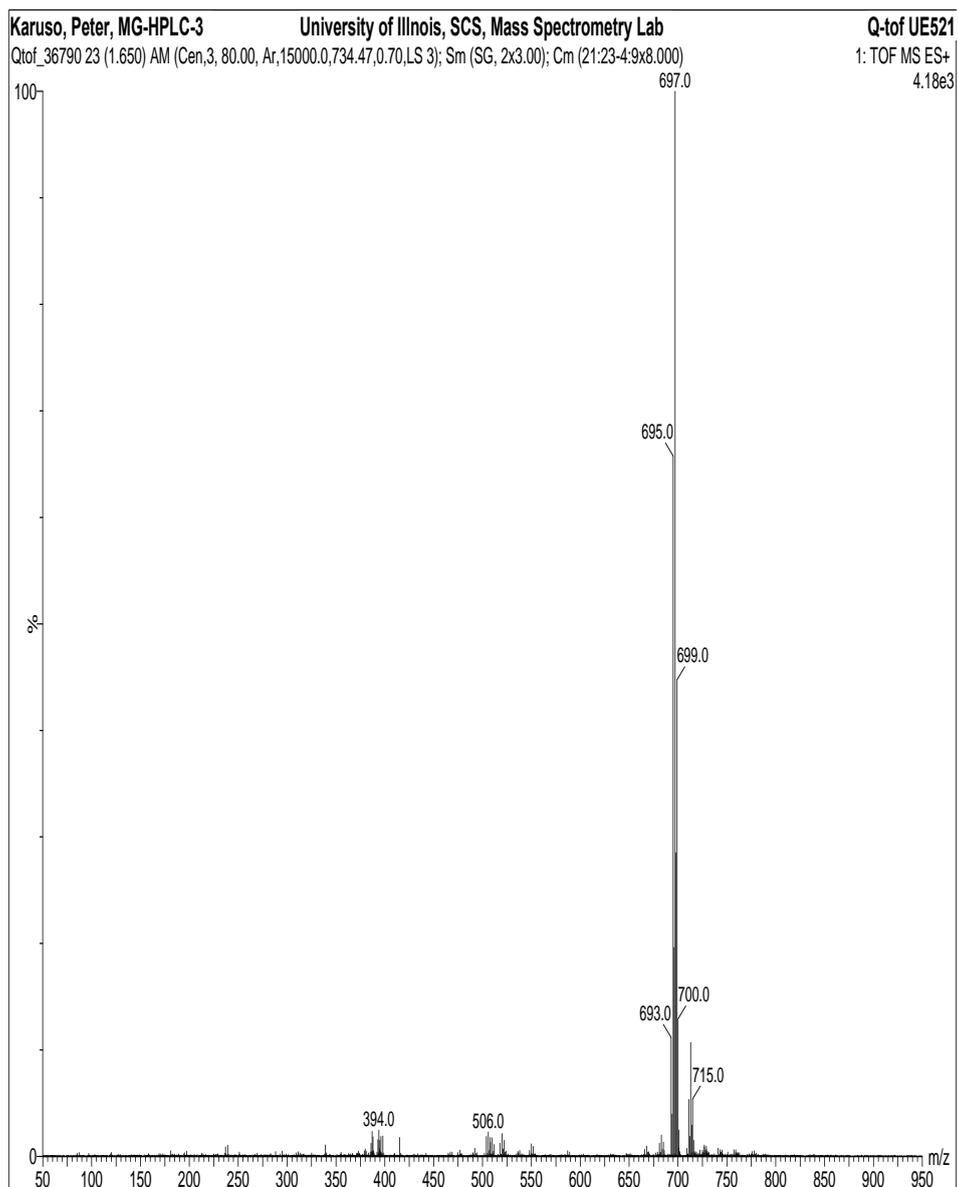


Figure S2. High resolution MS analysis of ceratinadin D (1).

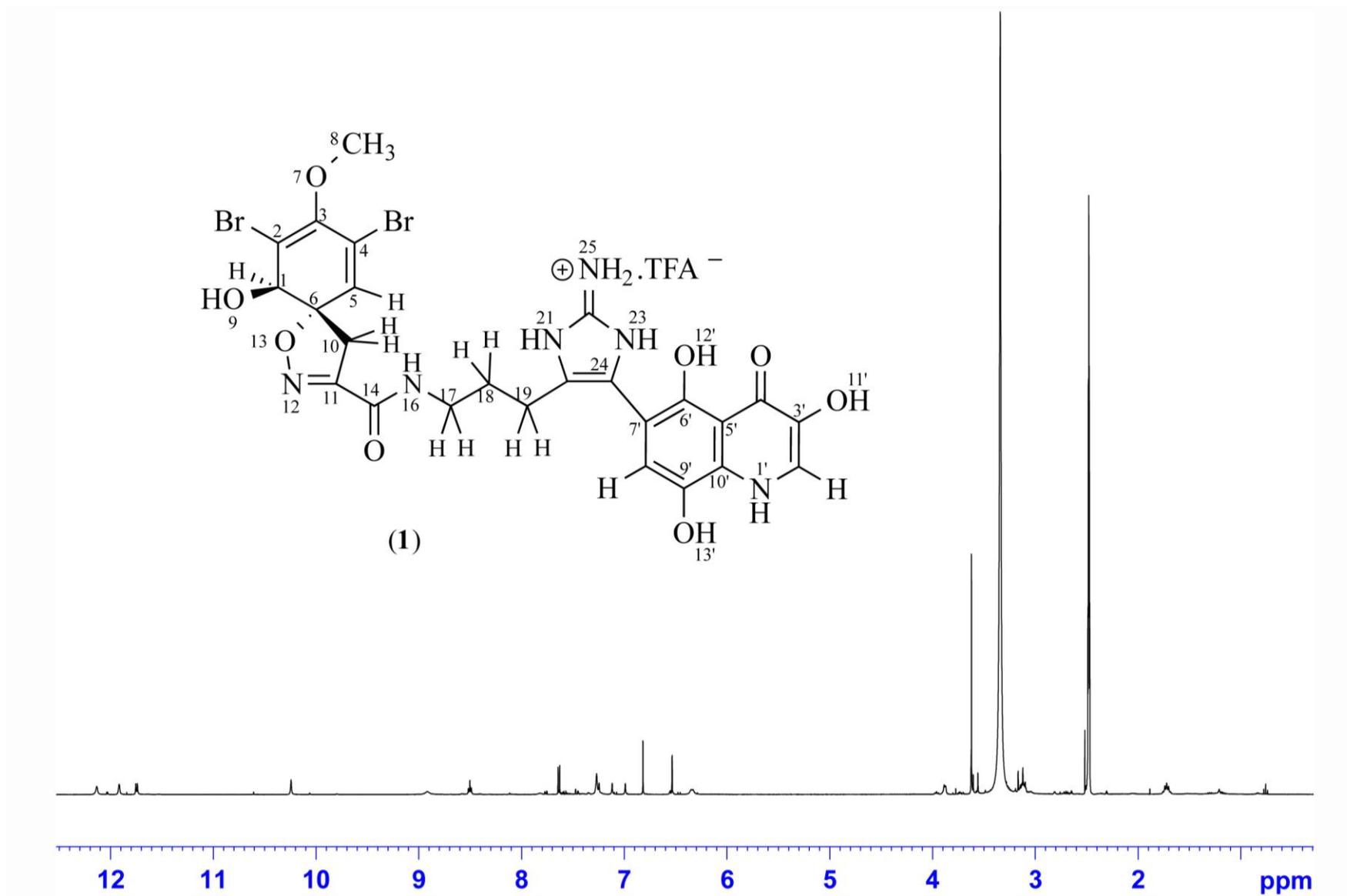


Figure S3. ¹H-NMR spectrum of ceratinadin D (1; DMSO-*d*₆, 600 MHz).

S1.6. Aplysamine 8 (2)

The high resolution MS report for the new bromotyrosine derivative aplysamine 8 (2) is shown in Figure S4. The $^1\text{H-NMR}$ spectrum of (2) is shown in Figure S5.

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 150.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

228 formula(e) evaluated with 3 results within limits (all results (up to 1000) for each mass)

Elements Used:

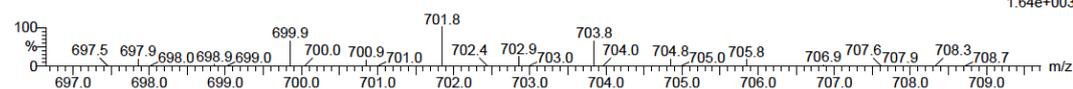
C: 0-150 H: 0-200 N: 0-5 O: 0-8 Br: 4-4

Karuso, Peter, MG-HPLC-10

University of Illinois, SCS, Mass Spectrometry Lab

Q-tof UE521

Qtof_36791 22 (1.578) AM (Cen,3, 80.00, Ar,15000.0,734.47,0.70,LS 3); Sm (SG, 2x3.00); Cm (21:22)

1: TOF MS ES+
1.64e+003

Minimum: -1.5
Maximum: 150.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
697.8527	697.8540	-1.3	-1.9	13.5	0.7	C26 H24 N O2 Br4
	697.8500	2.7	3.9	9.5	0.7	C21 H24 N3 O4 Br4
	697.8460	6.7	9.6	5.5	3.2	C16 H24 N5 O6 Br4

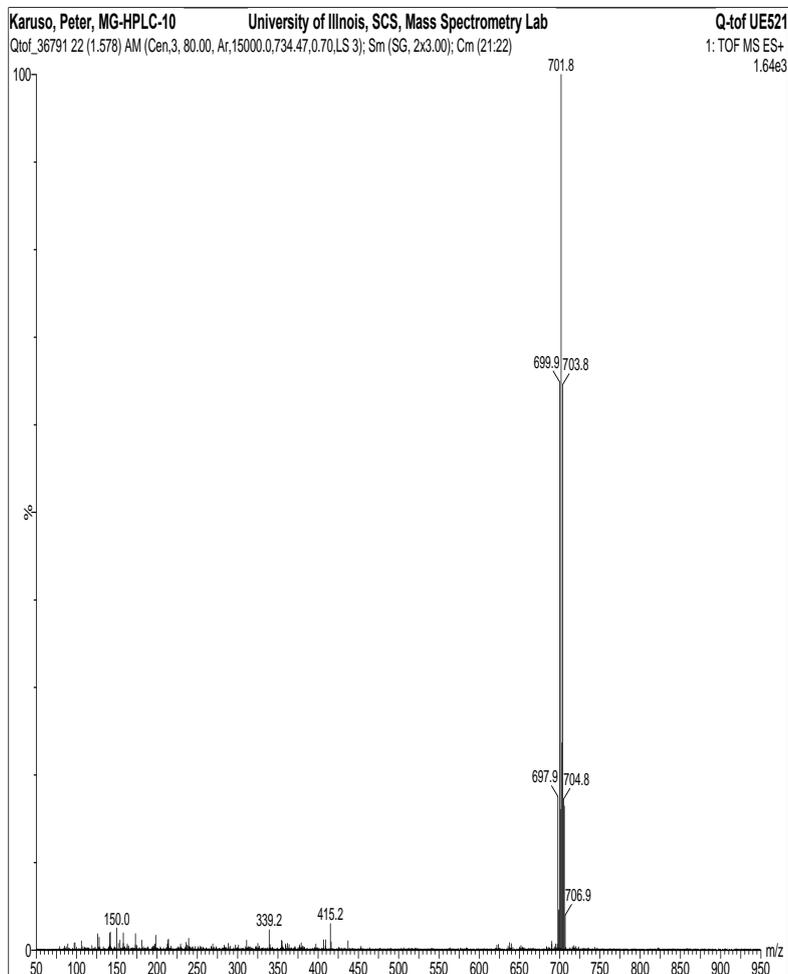


Figure S4. High resolution MS analysis of aplysamine 8 (2).

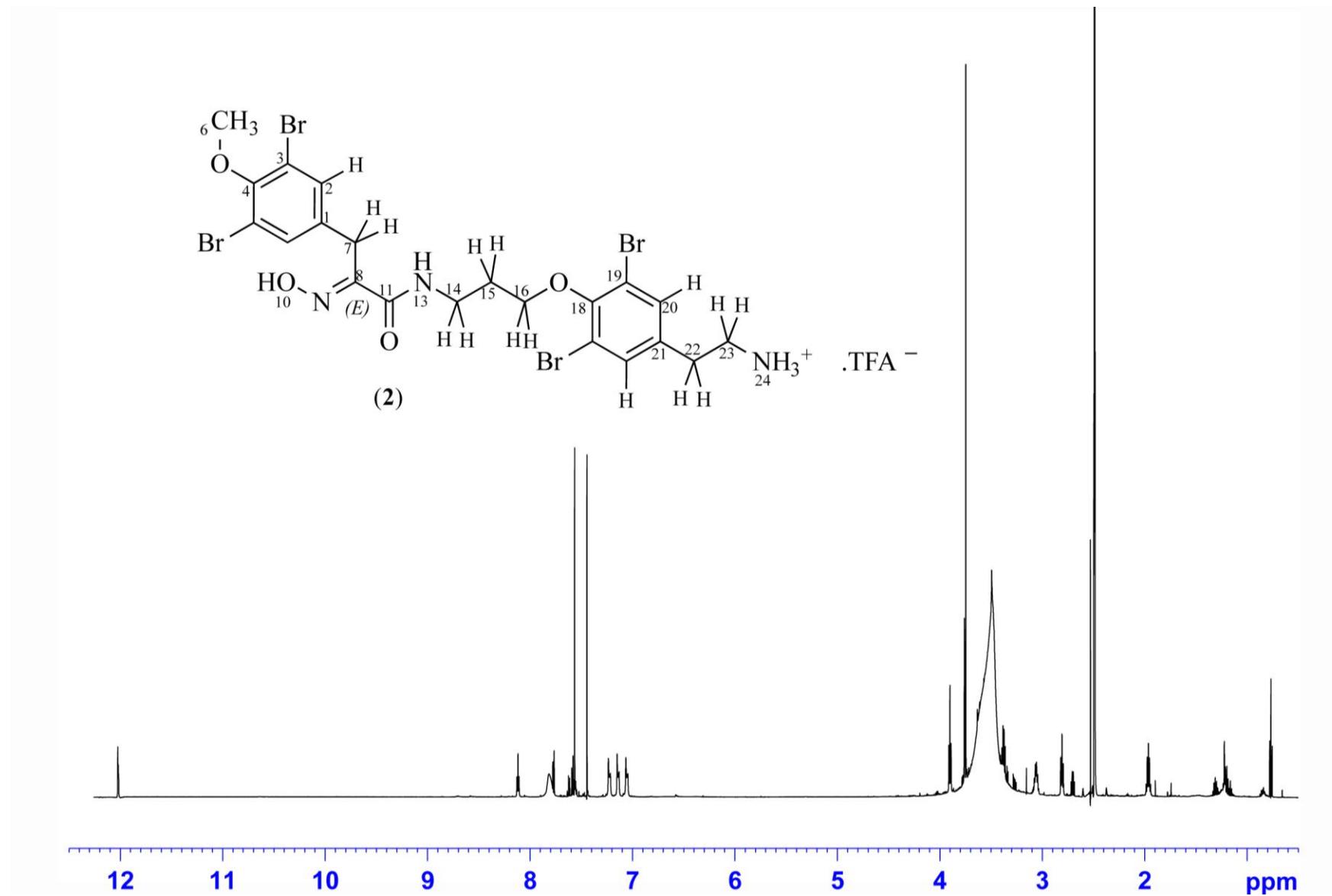


Figure S5. ¹H-NMR spectrum of aplysamine 8 (2; DMSO-*d*₆, 600 MHz).

References

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